ELSEVIER

Biochimica et Biophysica Acta 1465 (2000) 104–126

BIOCHIMICA ET BIOPHYSICA ACTA

BBA

www.elsevier.com/locate/bba

Review

Emerging mechanisms for heavy metal transport in plants

Lorraine E. Williams *, Jon K. Pittman, J.L. Hall

University of Southampton, School of Biological Sciences, Bassett Crescent East, Southampton SO16 7PX, UK

Received 1 November 1999; accepted 1 December 1999

Abstract

Heavy metal ions such as Cu^{2+}, Zn^{2+}, Mn^{2+}, Fe^{2+}, Ni^{2+} and Co^{2+} are essential micronutrients for plant metabolism but when present in excess, these, and non-essential metals such as Cd^{2+}, Hg^{2+} and Pb^{2+}, can become extremely toxic. Thus mechanisms must exist to satisfy the requirements of cellular metabolism but also to protect cells from toxic effects. The mechanisms deployed in the acquisition of essential heavy metal micronutrients have not been clearly defined although a number of genes have now been identified which encode potential transporters. This review concentrates on three classes of membrane transporters that have been implicated in the transport of heavy metals in a variety of organisms and could serve such a role in plants: the heavy metal (CPx-type) ATPases, the natural resistance-associated macrophage protein (Nramp) family and members of the cation diffusion facilitator (CDF) family. We aim to give an overview of the main features of these transporters in plants in terms of structure, function and regulation drawing on information from studies in a wide variety of organisms.

Keywords: CDF family; CPx-ATPase; Membrane protein; Nramp

1. Introduction

The mineral nutrition of higher plants is of fundamental importance to agriculture and human health, yet many basic questions remain unanswered, particularly in relation to the accumulation of essential heavy metals. How do plants ensure that all tissues receive an adequate supply of the heavy metals required for vital cellular processes yet prevent them from accumulating to toxic levels? This is a question of fundamental importance in plant biology and an area that is ripe for investigation now that the necessary molecular tools are available. Heavy metal ions such as Cu^{2+}, Zn^{2+}, Mn^{2+}, Fe^{2+}, Ni^{2+} and Co^{2+} are essential micronutrients for plant metabolism but when present in excess, these, and non-essential metals such as Cd^{2+}, Hg^{2+}, Ag^{2+} and Pb^{2+}, can become extremely toxic. For example, Cu^{2+} is an essential trace element that is involved in a number of electron transport reactions in both photosynthesis and respiration, while a wide range of enzymes either contain or are activated by Zn^{2+} and Mn^{2+} [1]. It has also been suggested that, in addition to calcium, some of these divalent cations may act as second messengers [2]. Thus, when these ions are not available to the roots, plants develop specific deficiency symptoms. At high concentrations, however, these metals can become extremely toxic, as do the non-essential metals, causing symptoms such as chlorosis and necrosis, stunting, leaf discolouration and inhibition of root

* Corresponding author. Fax: +44-1703-594319;
E-mail: l.e.williams@soton.ac.uk

0005-2736/00/$ – see front matter
PII: S0005-2736(00)00133-4

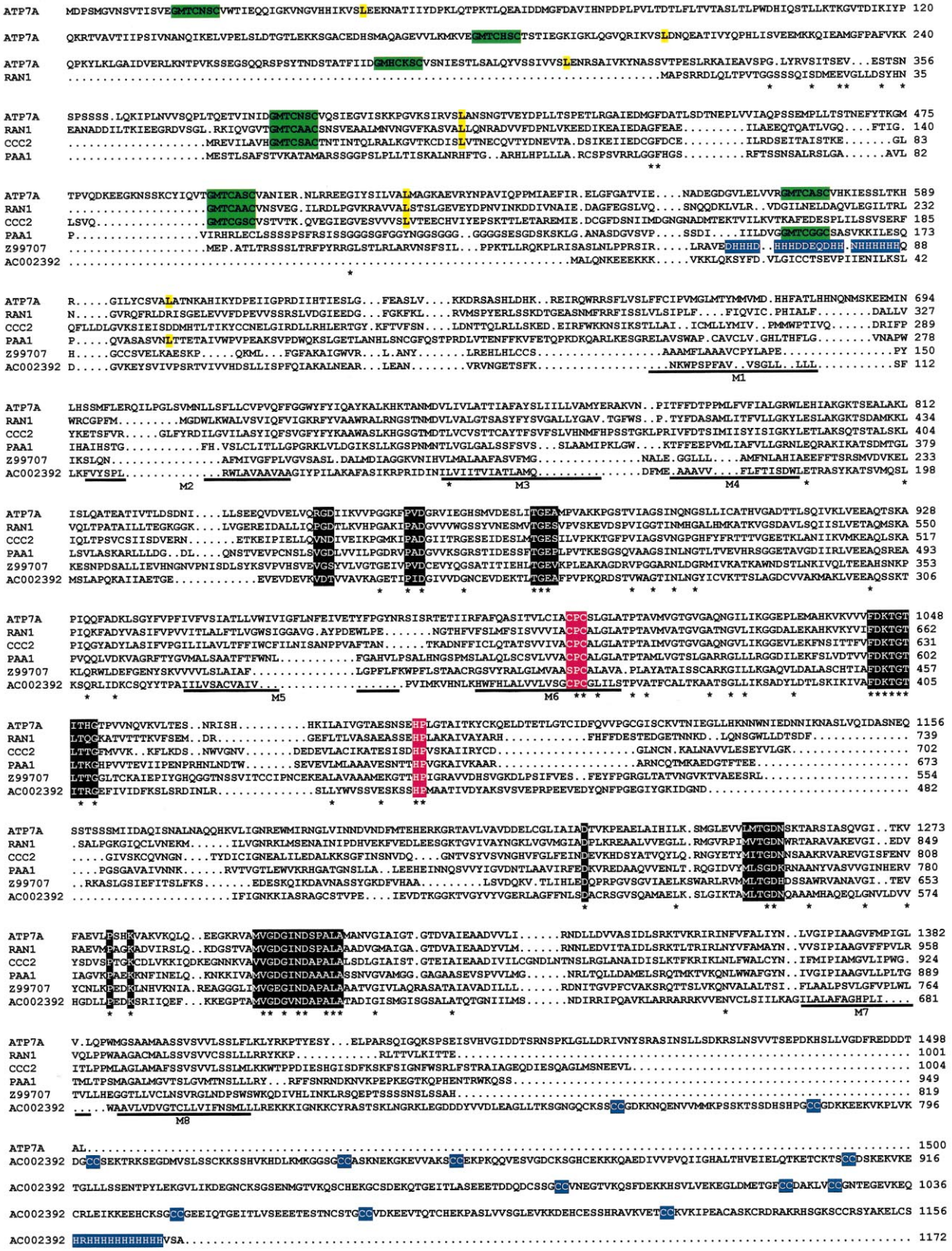
growth [1,3]. At the cellular level, toxicity may result from binding to sulfhydryl groups in proteins thereby inhibiting enzyme activity or protein function, or by producing a deficiency of other essential ions [3,4]. Other possibilities include disruption of cell transport processes and oxidative damage [4]. Thus the systems available for the acquisition of metal ions by the roots, transport and distribution around the plant, and regulation of their cytosolic concentrations are clearly integral to normal plant growth and development. By analysing the energetics of these processes, in many cases we can see that transport proteins must play a vital role in heavy metal homeostasis. The use of biochemical and molecular techniques will allow us to advance our knowledge of the transport proteins both in terms of their mechanistic properties and their physiological function. Tolerance to high concentrations of these metals in species and cultivars that can grow on metal-polluted soil could conceivably be achieved by excluding the uptake mechanisms from the root, or by efflux or compartmentation and detoxification of the metals following uptake. There is now considerable interest in the area of metal transport because of the implications for phytoremediation (defined as the use of green plants to remove pollutants from the environment or to render them harmless; [5]). However, our knowledge of the transport processes for heavy metals across plant membranes at the molecular level is still rudimentary in most cases. A comprehensive understanding of metal transport in plants will be essential for developing schemes to genetically engineer plants that accumulate specific metals, either for use in phytoremediation or to improve human nutrition [5]. This review will focus on several classes of proteins that have been implicated in heavy metal transport. It will centre on the role of heavy metal (or CPx-type) ATPases which have considerable potential as key heavy metal transporters in higher plants, involved not only in normal metal ion homeostasis but also in the overall strategy for heavy metal tolerance. In addition, the natural resistance-associated macrophage protein (Nramp) family of proteins will be discussed in relation to heavy metal transport, as will the cation diffusion facilitator (CDF) family. Certain heavy metal transporters have been reviewed by others in this issue and will not be covered here (see Guerinot, this volume).

2. Heavy metal-transporting ATPases: CPx-type ATPases

2.1. Introduction

P-type heavy metal ATPases have been identified in a wide range of organisms and have been implicated in the transport of a range of essential and also potentially toxic metals across cell membranes (e.g. Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}). They are a sub-group of the large superfamily of P-type ATPases which use ATP to pump a variety of charged substrates across biological membranes and are distinguished by the formation of a phosphorylated intermediate during the reaction cycle. A recent analysis [6] has shown that the various P-type ATPases group together into five major branches (types I–V) according to the transported substrate and not according to an evolutionary relationship of the parental species. For a more detailed discussion of other members of the P-type ATPases (Ca^{2+}-ATPases and H^{+}-ATPases), the reader is referred to other chapters in the issue. Heavy metal ATPases have been classified as type IB ATPases and, together with the closely related type IA ATPases (which are thought to be involved in K^{+} transport), they are considered to constitute a monophyletic group [7]. Type IB ATPases are found in bacteria, archaea and eukarya and are thought to have evolved early in evolution [7].

Solioz and Vulpe [8] have defined the heavy metal P-type ATPases as CPx-ATPases because they share the common feature of a conserved intramembranous cysteine-proline-cysteine, cysteine-proline-histidine or cysteine-proline-serine motif (CPx motif) which is thought to function in heavy metal transduction. They have been detected in species as diverse as bacteria, yeast and man. The family includes the two copper P-type ATPases implicated in Menkes disease (ATP7A) and Wilson's disease (ATP7B) which are disorders of copper metabolism and CCC2, a yeast CPx-ATPase functioning in copper transport into a post-Golgi compartment. The first CPx-ATPase reported in higher plants was PAA1 (P-type ATPase of *Arabidopsis* 1) from *Arabidopsis thaliana* but no functional data supporting a role in metal transport have yet been provided [9]. Recently, the exciting possibility was raised that the operation of the ethylene signalling pathway in


ATP7A
RAN1
CCC2
PAA1
Z99707
AC002392
M1
M2
M3
M4
M5
M6
M7
M8

plants requires a functional CPx-ATPase, RAN1 (responsive to antagonist 1) [10]. Functional evidence from yeast complementation studies suggested that RAN1 transports copper and it was proposed that this CPx-ATPase may have a role in delivering copper to the secretory system, required in the production of functional ethylene receptors [10]. Searching of the GenBank/EMBL sequence databases has suggested that there are other CPx-type ATPases in *Arabidopsis*; these will be referred to as AC002392 and Z99707, which are the accession numbers (Acc. No.) of the bacterial artificial chromosome (BAC) clones on which the genes were identified. The putative coding sequences for these genes are shown in Fig. 1. For Z99707, the prediction of part of the coding sequence is confirmed based on additional information from two expressed sequence tags (ESTs) covering the C-terminal and N-terminal region (Acc. No. Z33730 and Z33731). There are also ESTs corresponding to AC002392 (Acc. No. H26471, R90705, T04515, H36072, H36075) which confirm the protein prediction. Thus this information also indicates that these genes are expressed in *Arabidopsis*. Sequence database searching has also identified a cDNA sequence from soybean (Acc. No. AF019115) which encodes a partial-length protein that aligns with the C-terminal end of the CPx-ATPases. It has high sequence homology (76% identity) with Z99707, suggesting that it may be the homologous gene in soybean. Our studies in *Mimulus guttatus* have also indicated that several different heavy metal ATPases occur in this species (Evans, Williams and Hall, unpublished).

There is another class of P-type ATPase that may also have a role in heavy metal homeostasis. A subgroup of ATPases classified as type IV ATPases by Axelsen and Palmgren [6], identified in *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and some mammals, has been suggested to transport phospholipids. A key feature of this sub-group is the presence of strongly hydrophobic residues within the putative ion translocation domain. Recent experimental evidence using DRS2, a type IV ATPase from yeast, has shown that while this protein may be involved in transport of certain phospholipids, it may also be involved in either the direct transport or regulation of heavy metals in yeast [11]. This study found that a *drs2* deletion mutant was hypersensitive to some heavy metals, particularly zinc and cobalt, suggesting that DRS2 is required in some way to alleviate the toxic effect of certain metals in yeast [11]. At least two type IV ATPases are present in the *Arabidopsis* genome [7] and further work is required to determine whether this sub-group of ATPases may also have a role in metal regulation in this plant. This sub-family will not be discussed further in this review.

2.2. Structure

A schematic diagram comparing key features in a typical non-heavy metal ATPase (AtECA1) and a putative heavy metal CPx-ATPase (PAA1) from *A. thaliana* illustrates the similarities and differences in the two types (Fig. 2). Generally, CPx-type ATPases share many features common to all P-type ATPases although they also have some unique characteristics (see [8] for detailed discussion). They are similar in that a large proportion of the pump, comprising the large and small cytoplasmic domains, protrudes into the cytoplasm. Most of the CPx-ATPases analysed to date also contain an N-terminal cytoplasmic domain thought to be involved in metal-binding. They also differ in the number and topology of the membrane-spanning domains (see Section 2.2.1).

←

Fig. 1. Multiple alignment of the putative CPx-type ATPases from *Arabidopsis* with the human Menkes disease Cu^{2+}-ATPase ATP7A [96] and the yeast homologue CCC2 [89]. The alignment was performed using ClustalW (1.7) sequence alignment software then manually edited. An asterisk below the sequence indicates identical amino acids. The putative transmembrane helices (M1–M8) for PAA1 are underlined. Motifs strongly conserved in all P-type ATPases are highlighted in black. Motifs unique to CPx-type ATPases are highlighted in red. CxxC-type metal-binding motifs are highlighted in green. Other possible metal-binding regions are highlighted in blue. Conserved leucine residues associated with metal-binding domains are highlighted in yellow; see text for further discussion. The accession numbers and references for the sequences used are as in Table 1. Sequences were obtained from the EMBL/GenBank databases.

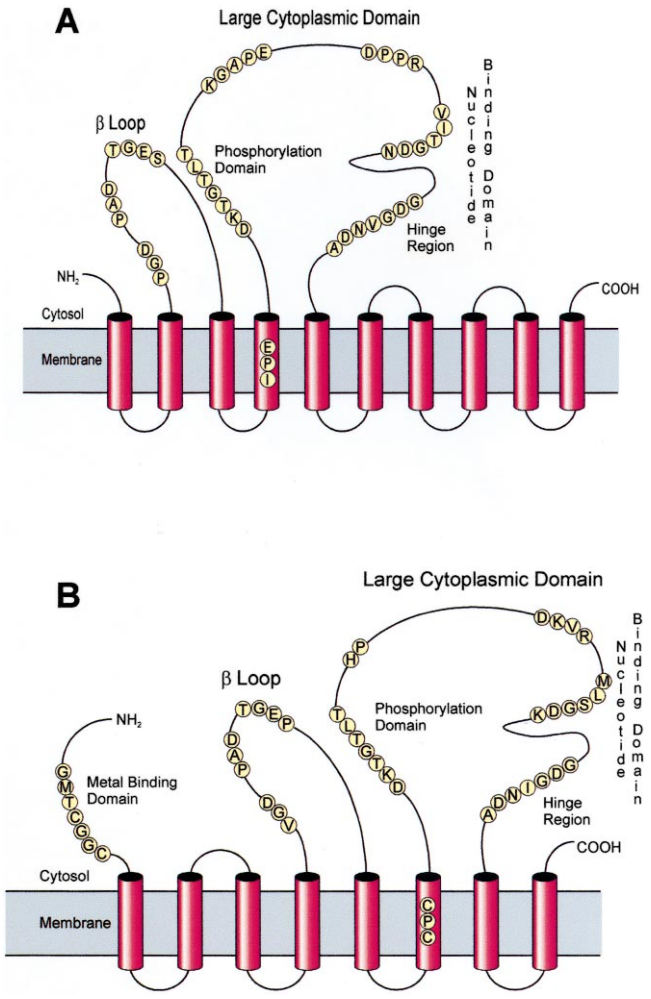


Fig. 2. A structural model comparing two types of P-type ATPase from *Arabidopsis*. (A) shows a Ca^{2+}-ATPase (AtECA1; [98]) while (B) shows a putative CPx-type ATPase (PAA1; [9]). Putative transmembrane helices are depicted as red cylinders and the amino acid residues of conserved motifs are shown.

2.2.1. Topology

The membrane architecture derived from the sequences of the heavy metal ATPases does not follow that of the other P-type ATPases. The membrane topology can be predicted by hydropathy analysis and we have compared the CPx-ATPases PAA1, RAN1, Z99707 and AC002392 from *Arabidopsis* with the Menkes CPx-ATPase, ATP7A, and also AtECA1, a type IIA Ca^{2+}-ATPase from *Arabidopsis* (Fig. 3). The hydropathy plots were generated from the amino acid sequences using the Kyte–Doolittle algorithm [12]. The CPx-ATPases are novel in having four transmembrane domains before the first cytoplasmic domain as opposed to the two seen for the non-heavy metal ATPases. In addition, following the second cytoplasmic domain they are predicted to have two transmembrane domains, whereas in other P-type ATPases there can be four or more transmembrane domains in this region. Characteristically, the CPx motif is found in transmembrane domain 6. Obviously, this discussion is only based around predictions from hydropathy analysis and experimentation will be required to confirm the exact topology. Another notable feature is that generally the large cytoplasmic loop is smaller for the CPx-ATPase than for the other P-type ATPases. Between the phosphorylation domain and the hinge region, the *Arabidopsis* CPx pumps have approximately 150 amino acid residues less than the *Arabidopsis* Ca^{2+} pump, AtECA1. This feature was used to distinguish

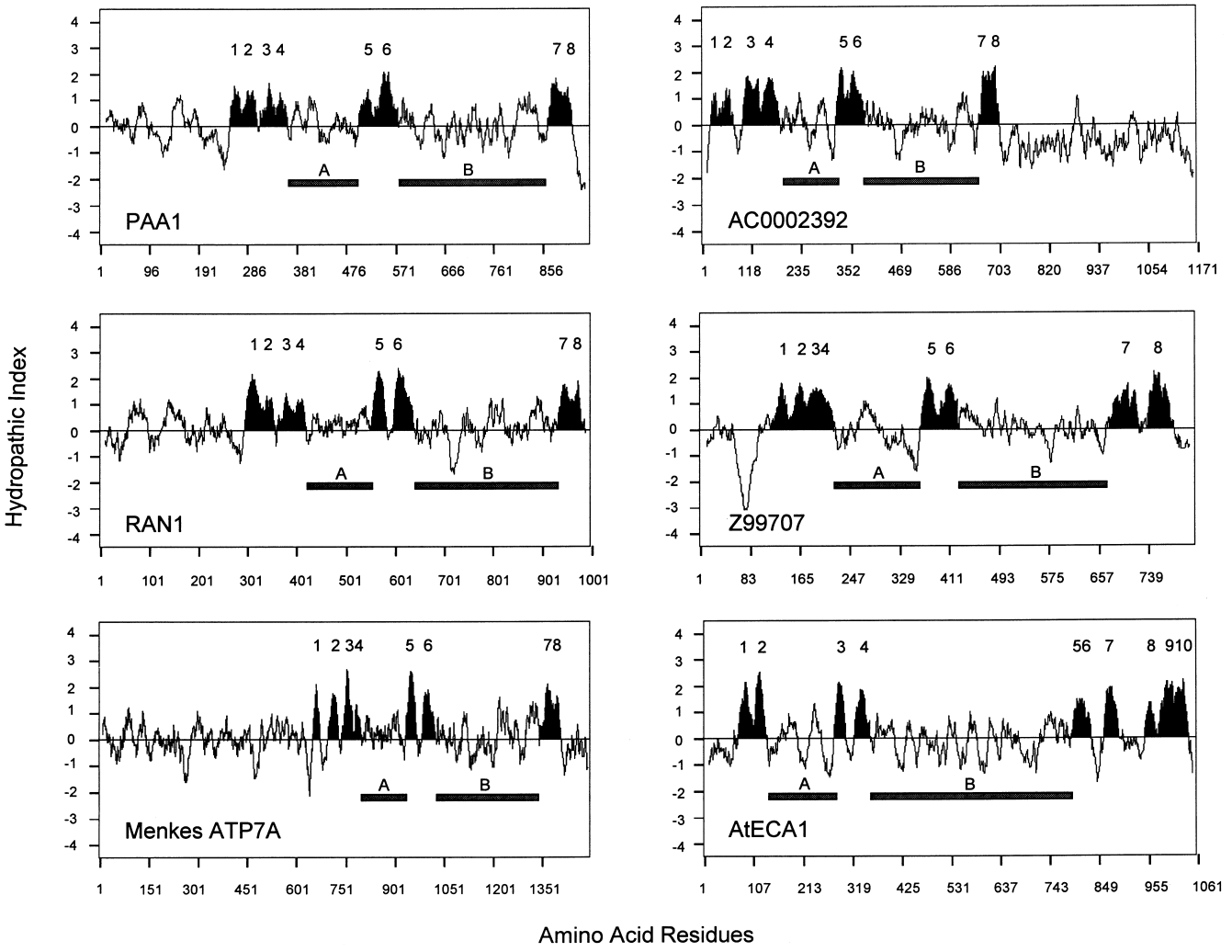


Fig. 3. Hydropathy plots of putative CPx-type ATPases from *Arabidopsis*, compared with the human Menkes disease Cu^{2+}-ATPase ATP7A and the Ca^{2+}-ATPase AtECA1 from *Arabidopsis*. Plots were calculated according to Kyte and Doolittle [12] with a window size of 21 amino acids. The predicted transmembrane regions are shaded. Grey bars denote the β-loop domain (A) and the large cytosolic loop domain (B).

between ATPases when the *PAA1* gene was identified by PCR [9].

2.2.2. Comparison of conserved motifs

Like all P-type ATPases, the CPx-ATPases possess the **D**KTGTLT motif containing the aspartate residue which is phosphorylated by ATP in the reaction cycle [13,14]. They also contain a conserved motif in the hinge region GDGxNDx (GDGVNDA in AtECA1 and GDGINDA in PAA1; Fig. 2). These two motifs are amongst the most conserved in P-type ATPases throughout evolution [6]. The GDGxNDx motif has been implicated in ATP-binding, and mutagenesis studies have shown that it is important for the function of the ATPase [13]. Two of the residues which are mutated in Wilson's disease protein (ATP7B) are present in this motif (**G**DGx**N**Dx), indicating its importance for the CPx-ATPases [14]. In addition, the two conserved aspartate residues of this motif have been proposed to have a role in hydrolysis of the acylphosphate intermediate [14]. Both of these motifs are found in the large cytoplasmic loop domain that also includes another conserved region, the T**GD** motif (VIT**GD**N in AtECA1 and MLS**GD**K in PAA1; Fig. 2). This motif (particularly the glycine and aspartate residues) was initially im-

plicated as forming part of the ATP-binding domain but may also be involved in phosphorylation rather than ATP-binding [15,16]. This aspartate residue is also mutated in the Wilson's disease protein [14]. The KGAPE motif and the DPPR motif seen in AtECA1 and other P-type ATPases are not evident in the CPx-ATPases (Figs. 1 and 2). However, the aspartate of **D**PPR is present in all these ATPases including the CPx-type. The PxxK motif is also conserved amongst all P-type ATPases including the CPx-type [6,14]. It has been suggested that the conserved lysine of this motif may be involved in charge stabilisation [14].

Sequences conserved in the smaller cytoplasmic loop (also referred to as the β-strand domain or the β-domain loop; [13]) include the PGD, PAD and TGES motifs, although the PGD and PAD motifs are not as conserved amongst CPx-ATPases as they are in other P-type ATPases. This region may be involved in energy transduction [13]. The importance of the TGES motif for CPx-ATPases is highlighted for the *Arabidopsis* Cu^{2+}-ATPase, RAN1 [10]. A partial loss of function mutant *ran1-1* was identified which contained a single amino acid change whereby Thr^{497} of **T**GES is changed to Ile. The *ran1-1* mutant was unable to functionally complement the yeast *ccc2*Δ deletion mutant, demonstrating that RAN1 is unable to transport copper without a functional TGES motif [10].

All P-type ATPases contain a conserved proline in the transmembrane domain preceding the large cytoplasmic loop 43 amino acids N-terminal to the phosphorylatable aspartate [13]. The amino acids surrounding the proline vary with the ion specificity of the transporter and in the Ca^{2+}-ATPase, AtECA1, the sequence is I/VPE. However, in the CPx-ATPases, there is a cysteine to the amino-terminus and either a cysteine, histidine or serine to the C-terminus, hence leading to their classification as CPx-ATPases [8]. In CPx-ATPases, this region is thought to play an important role in the translocation of heavy metals [13,17]. In the large cytoplasmic loop of heavy metal ATPases, there is also a conserved HP locus which is not found in non-heavy metal ATPases. This is found 30–50 amino acids C-terminal to the phosphorylated aspartate residue and it is suggested to play a fundamental structural role in the function of CPx-ATPases, possibly as a site for protein–protein interaction [18]. The percentage identity between the plant sequences is not particularly high (Table 1) and although they all contain the conserved P-type ATPase motifs and the HP locus (Fig. 1), Z99707 contains the motif SPC instead of CPC; this also occurs in *Synechocystis* PC6803 CadA [19].

2.2.3. Metal-binding motifs

Other unique features of CPx-ATPases (in addition to the CPx motif) include one or several putative metal-binding motifs in the N-terminal region preceding the first predicted transmembrane domain. In many cases, this consists of one or several cysteine-rich CxxC motifs. In the human Menkes

Table 1
Sequence similarities between heavy metal P-type ATPases

	PAA1	Z99707	AC002392	RAN1	CCC2	ATP7A	ZntA	CadA
Species	*A. thaliana*	*A. thaliana*	*A. thaliana*	*A. thaliana*	*S. cerevisiae*	*Homo sapiens*	*E. coli*	*Synechocystis*
Substrate	nd	nd	nd	Cu	Cu	Cu	Pb/Zn/Cd	Cd
PAA1		27.1	29.9	33.0	32.8	33.0	34.2	34.9
Z99707	34.0		28.6	27.2	26.0	27.9	31.2	31.6
AC002392	38.3	38.4		32.1	25.5	30.3	32.6	31.6
RAN1	39.1	35.7	42.5		39.9	41.2	33.1	34.3
CCC2	41.1	35.9	38.4	51.2		37.1	28.9	29.2
ATP7A	41.9	37.9	42.1	51.2	48.6		33.1	37.5
ZntA	42.5	39.7	42.4	43.7	38.3	43.7		39.5
CadA	45.3	42.9	44.6	44.2	42.1	49.0	50.5	

Percentage identities (upper right triangle) and percentage similarities (lower left triangle) were calculated using the GAP program (GCG software package). Accession numbers and references for published sequences are: PAA1 (D89981; [9]), RAN1 (AF082565; [10]), CCC2 (P38995; [97]), ATP7A (Q04656; [96,101]), ZntA (P37617; [34]), CadA (D64005; [19]).
nd, not determined.

Cu^{2+}-ATPase, there are six GMTCxxC sequences whereas in PAA1 and RAN1 from *Arabidopsis*, there are one and two GMTCxxC motifs, respectively (Fig. 1). It should be stressed that the GMTCxxC motif is also found on other metal-binding proteins which are not CPx-ATPases (e.g. the metal chaperone proteins; see Section 2.4). In certain CPx-ATPases, e.g. HRA1 and HRA2 of *Escherichia coli* and CopB of *Enterococcus hirae*, instead of the CxxC motif there are methionine- and histidine-rich sequences which are thought to be metal-binding domains [8]. The *Arabidopsis* putative heavy metal ATPase, Z99707, does not have any CxxC motifs but has a number of histidines in the N-terminal region which may be involved in metal-binding (Fig. 1). Histidine residues are commonly found on zinc-binding motifs, particularly those found on zinc finger proteins and zinc RING finger proteins [20]. Histidine-rich motifs, particularly with the consensus motif HxHxH, are present on other zinc transport proteins, including members of the ZIP family [21] and members of the CDF family ([22]; see below), indicating that zinc may be a possible substrate for this ATPase. However, the HxHxH motif is also found on proteins that bind metals other than zinc, and it has been suggested that these motifs may be involved in metal-regulated protein–protein interactions [21]. AC002392 is also unusual in not having the typical binding motifs in the N-terminus. Instead it has a C-terminal region with multiple putative metal-binding motifs rich in histidine and cysteine with cysteine residues found as CC pairs and a stretch of repeated histidine residues at the end (Fig. 1).

There is now good evidence that the GMTCxxC motif does have a role in metal-binding and in ATP7B, it has been shown to have specificity for copper with a stoichiometry suggesting that each metal-binding domain binds one copper atom [23]. The functional role of the metal-binding domains in CPx-ATPases is still not clear. They may capture ions and transfer them to the membrane-associated translocation domain or possibly have storage or regulatory roles [24]. The putative copper-binding domains may not be functionally equivalent [18,25]. A recent study looked at the role of all six binding domains on the Menkes disease protein [25]. This ATPase is normally located at the Golgi but is redistributed to the plasma membrane during high copper conditions (see Section 2.5). This copper-induced response was used to determine whether all six metal-binding domains are required for copper-induced trafficking [25]. It was found that the protein could function without the first four metal-binding domains and just one of the domains nearest to the membrane (either domain five or six) was sufficient. The putative Cu^{2+}-ATPases from yeast and *Arabidopsis* have only one or two GMTCxxC domains, suggesting that this is sufficient for the basic function. Additional metal-binding domains found on the Menkes and Wilson's disease proteins may improve efficiency of the ATPase in some way.

The solution structure of the fourth metal-binding domain from the Menkes protein has been determined [24]. Using Ag(I) as the binding metal, it was confirmed that the two cysteine residues in the $GMTCxSC^{502}$ motif interact with the metal ion. The metal-binding pocket is defined by the side chains of both cysteine residues as well as the side chains of Thr^{498} and Ser^{501} [24]. This serine residue is conserved in all six metal-binding domains of both Menkes and Wilson's ATPases while PAA1 and RAN1 have a glycine and alanine substitution, respectively, at this position in their metal-binding domains (Fig. 1). Both of these residues have short side chains, therefore metal-binding in this pocket should still occur, although binding affinities of the metal ion may differ.

The GMTCxxC metal-binding motif is also present in mercury, cadmium- and zinc-binding domains [8,24]; therefore, other residues may be involved in determining the metal specificity or affinity of a particular ion to the binding site. A leucine residue, 21 residues from the CxxC motif, present in all six binding domains of Menkes and Wilson's proteins is also present in all copper transporters ([24]; highlighted in yellow in Fig. 1). This residue is packed beneath the metal-binding loop (in contact with the first cysteine residue of the **C**xxC motif) and it may be involved in conferring substrate specificity to the metal-binding domain. In contrast, mercury-, cadmium- or zinc-binding domains have a phenylalanine or tyrosine residue instead of leucine at this position [24]. The *Arabidopsis* RAN1 and PAA1 ATPases have a leucine residue in this position following their putative metal-binding motifs (Fig. 1). A *ran1-2* mutant of the *Arabidopsis* Cu^{2+}-

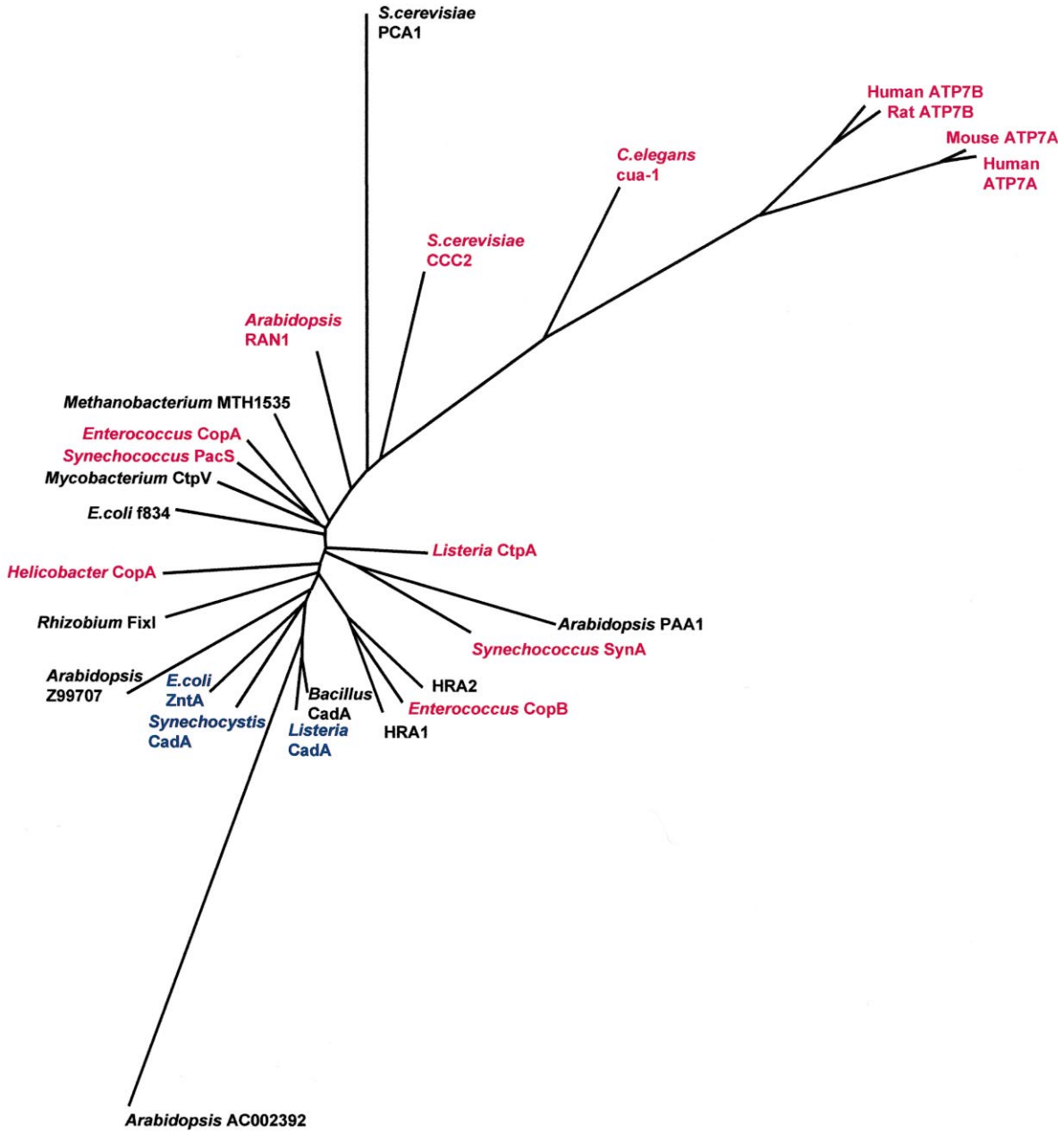


Fig. 4. Phylogenetic analysis comparing the CPx-type ATPases from *Arabidopsis* with those from a range of animals, fungi and bacteria. The tree was constructed from alignments of full-length amino acid sequence of each ATPase using the Protdist and Fitch programs on the PHYLIP package [99]. For ATPases highlighted in red, there is functional evidence for copper transport. For ATPases highlighted in blue, there is functional evidence for cadmium, zinc or lead transport. The accession numbers for the sequences used are: human ATP7A Q04656, human ATP7B P35670, mouse ATP7A U03434, rat ATP7B U08344, cua-1 D83665, CCC2 P38995, PCA1 P38360, MTH1535 AE000913, *Enterococcus* CopA P32113, PacS P37297, CtpV P77894, f834 Q59385, *Helicobacter* CopA P77871, FixI P18398, CtpA U15554, PAA1 D89981, RAN1 AF082565, SynA P37385, *Enterococcus* CopB P05425, HRA1 U16658, HRA2 U16659, ZntA P37617, *Synechocystis* CadA D64005, *Listeria* CadA Q60048, *Bacillus* CadA P30336. Sequences were obtained from the EMBL/GenBank or SwissProt databases.

ATPase was identified which has a single amino acid substitution from Gly^{173} to Glu [10]. In the wild-type RAN1, Gly^{173} is found five residues from the conserved leucine (Leu^{168}) in the second metal-binding domain (Fig. 1). Complementation of the yeast *ccc2*Δ deletion mutant with *ran1-2* was less successful than complementation with RAN1. It was suggested that the Gly^{173} mutation disrupted the second metal-binding domain, and although *ran1-2* was able to complement the yeast mutant with a single functional metal-binding domain, the copper transport activity was reduced [10].

2.3. Phylogenetic analysis

A comparison between the amino acid sequences of the type IB heavy metal ATPases can be used to determine their phylogenetic relationships. Fig. 4 shows a phylogenetic tree for CPx-ATPases derived from an alignment of full-length amino acid sequences. The ATPases highlighted in red have been shown to transport copper or have sequence homology with copper-transporting ATPases. Some of the other ATPases have been shown to transport cadmium (highlighted in blue) or other metals including zinc or lead (for ZntA [26]). The *Arabidopsis* proteins do not cluster in one group, suggesting that they may have different transport functions. PAA1 and RAN1 cluster more closely with proteins which have been shown to transport copper, consistent with the functional evidence for copper transport by RAN1 [10] and suggesting that PAA1 may also transport this metal. Z99707 clusters more closely to the cadmium-transporting ATPases as does AC002392. However, direct transport measurements must be performed before a particular transport function can be assigned to the plant proteins.

2.4. Function

CPx-ATPases are thought to be important not only in obtaining sufficient amounts of heavy metal ions for essential cell functions but also in preventing accumulation of these ions to toxic levels. The human Menkes disease gene encodes a copper pump with defects resulting in copper build-up in some tissues. Most CPx-ATPases identified to date have been implicated in copper or cadmium transport although zinc tolerance in *E. coli* and *Synechocystis* PCC 6803 has been linked to CPx-type ATPases that export zinc from the cytosol [27,28]. The demonstration of transport function has been achieved only for some of the heavy metal ATPases. For example, ATPase activity, acylphosphate formation and copper transport have been demonstrated for the CopB ATPase of *E. hirae* [17,29] while the Menkes protein (ATP7A) has been shown to form an acylphosphate intermediate [30] and to transport copper [31]. CadA of *Staphylococcus aureus* has been shown to catalyse ATP-coupled, vanadate-sensitive cadmium transport [32] and to form a phosphorylated intermediate [33] while ZntA from *E. coli* has been shown to carry out ATP-dependent, vanadate-sensitive transport of zinc and cadmium [34]. Recently, Rensing et al. [26] demonstrated that CadA and ZntA could also transport lead and thus function physiologically in providing resistance to environmental lead. Caution is needed in assuming that the substrate transported in vivo is the same as that observed from the in vitro studies. Indeed, it is important that we ascertain the concentrations of metal ions that the transporter may experience in planta before we draw conclusions concerning the physiological substrate.

We can only speculate on the possible physiological roles of the heavy metal ATPases in higher plants, especially as the substrate specificity is unknown for most of the plant homologues. Successful complementation of the yeast *ccc2*Δ deletion mutant by RAN1 indicates that this ATPase functions in copper transport ([10]; see below); however, ATP-dependent copper transport by this protein has yet to be demonstrated directly. Despite the presence of certain conserved sequence motifs, the amino acid sequences of the plant CPx-ATPases are quite variable in other regions. This may suggest that there are differences in the function of these enzymes. However, the sequence identity between all CPx-ATPases is very low (on average 30–40%), including those which have been shown to transport the same metal (Table 1). As well as for RAN1, there is functional evidence for another putative heavy metal transporter in plants, AMA1 (*Arabidopsis* heavy metal ATPase 1) [35]. In this case, the heavy metal content was analysed in seedlings of a T-DNA knockout of *AMA1* (*ama1-1*), and it was found that molybdenum was the only essential micronutrient to show a reduc-

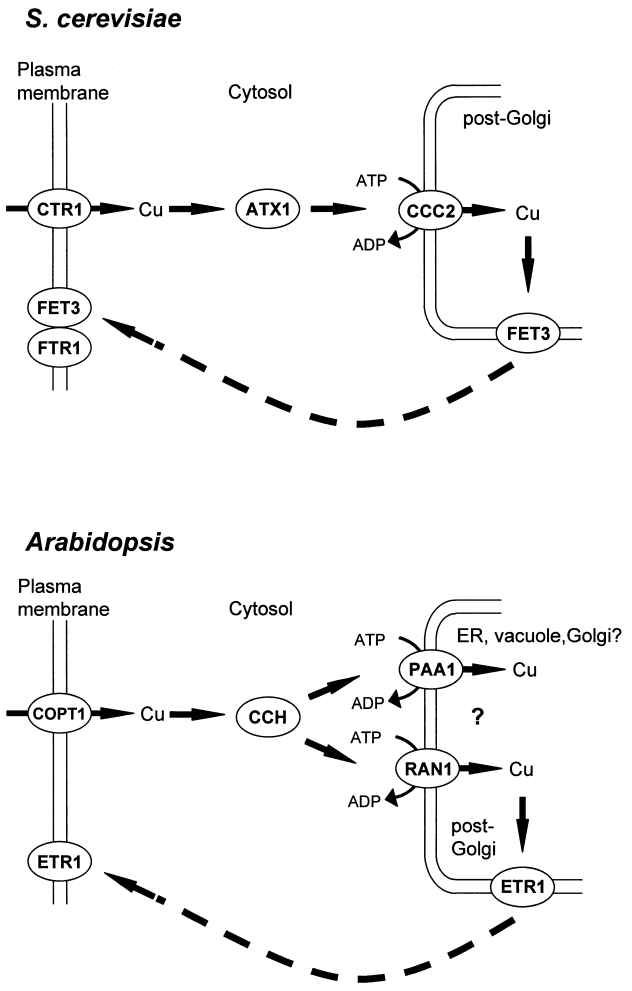


Fig. 5. Proposed pathways for copper trafficking in yeast and *Arabidopsis* cells. In yeast, CTR1 transports copper across the plasma membrane where it is transferred to the soluble copper chaperone ATX1. ATX1 physically interacts with the CPx-ATPase, CCC2, which pumps copper into a post-Golgi secretory compartment where it is inserted into a multicopper oxidase FET3 which is then targeted to the plasma membrane where it associates with the iron permease FRT1. For *Arabidopsis*, a hypothetical pathway is shown based on the existence of plant homologues for the yeast trafficking proteins. COPT1 could serve to transport copper across the plasma membrane. The putative copper chaperone CCH may then transfer copper to a CPx-ATPase such as PAA1 or RAN1 which pumps copper into an intracellular compartment. In a post-Golgi compartment, RAN1 is proposed to insert copper into an ethylene receptor such as ETR1 [10]. The insertion of copper results in a functional ethylene receptor which is then targeted to the plasma membrane where it is able to perceive ethylene.

tion in levels compared to wild-type controls. AMA1, detected using two different polyclonal antibodies to N- and C-terminal domains, was enriched in the plasma membrane fraction and was found to show highest levels in the root and flowers. Thus, in roots, AMA1 may function in molybdenum transport [35]. Conceivably, such a pump could exist at the xylem parenchyma and function in pumping heavy metal ions into the xylem for transport to the rest of the plant.

Since the other *Arabidopsis* CPx-ATPases show fairly low similarity to each other, it is possible that they transport different substrates. They may be present at the plasma membrane and function as efflux pumps removing potentially toxic metals from the cytoplasm or they may also be present at various intracellular membranes and be responsible for compartmentalisation of heavy metals, e.g. sequestration in the vacuole, Golgi or endoplasmic reticulum (ER).

CPx-ATPases may also play a role in trafficking of metal ions. There have been some very interesting developments in the area of copper trafficking in yeast and these may give an indication of the processes operating in higher plants. Indeed, homologues of some of the proteins involved in copper trafficking in yeast have now been found in *Arabidopsis*, warranting a brief discussion of the situation (for a full account, see [36,37]). In yeast, copper is transported across the plasma membrane by the transport proteins CTR1 and CTR3, and is then bound to cytosolic copper chaperone proteins. One such copper-binding chaperone is ATX1 [38], which contains a single N-terminal CxxC metal-binding domain. Copper is delivered to the secretory system following interaction of ATX1 and the vesicular copper CPx-ATPase, CCC2 (Fig. 5). The copper is inserted into a multicopper oxidase, FET3, which is essential for high-affinity iron uptake. FET3 is an integral membrane protein that mediates iron oxidation, resulting in the transport of iron across the plasma membrane through the permease FTR1 [39,40]. A homologue of ATX1 has now been found in *Arabidopsis* (CCH, copper chaperone) which restores high-affinity iron uptake to yeast mutants [41]. In addition, COPT1 (copper transporter 1), a putative copper transporter from *Arabidopsis* [42], shows some sequence similarity to CTR1; thus a similar pathway may operate in *Arabidopsis* with

one of the CPx-ATPases playing an analogous role to CCC2 ([42,43]; see Fig. 5). In this respect, the yeast CCC2 deletion mutant (*ccc2*Δ) is a useful model system for investigating putative Cu^{2+}-ATPases from other organisms including higher plants. Successful complementation of this yeast mutant has indeed been achieved with Cu^{2+}-ATPases from human [18], *C. elegans* [44] and *Arabidopsis* [10].

There is now evidence that the *Arabidopsis* CPx-ATPase, RAN1, may have a similar role to CCC2 and play an important role in the copper trafficking pathway in plants [10]. In a recent study, the importance of RAN1 in the ethylene signalling pathway has been demonstrated. Several important pieces of data which will be outlined here were presented providing evidence that RAN1 may function in delivering copper ions to create a functional ethylene receptor [10]. It is thought that normally, in the absence of ethylene, the ethylene receptors are active and function to negatively regulate downstream signaling pathway components, preventing hormone response phenotypes. Binding of ethylene inactivates the receptors and the ethylene response pathway is activated. RAN1 was identified in a mutant-based screening strategy to identify important components in the ethylene signalling pathway. Two mutants, *ran1-1* and *ran1-2*, were isolated in a screen using the ethylene receptor antagonist, *trans*-cyclooctene (TCO). Following exposure to this compound, these mutants showed a similar response to that seen when wild-type plants are exposed to ethylene ('ethylene' triple response phenotype). These results suggested that in these mutants, the antagonist was in fact acting as an agonist and mimicking the effects of ethylene. Growth on copper-supplemented media prevented *ran1* seedlings from responding to TCO (i.e. no triple response phenotype), indicating that the phenotype of *ran1* mutants was caused by a defect in copper metabolism. Results with transgenic plants indicated that a reduction in *RAN1* expression results in constitutive expression of ethylene responses. Complementation of the yeast deletion mutant, *ccc2*Δ, with RAN1 from *Arabidopsis* was assayed by measuring both FET3 oxidase activity and high-affinity iron uptake. Complementation by RAN1 increased FET3 activity and iron uptake to almost wild-type levels for the yeast [10], showing that this ATPase is able to transport copper in the yeast cell. From this range of information, Hirayama et al. [10] proposed that RAN1 functions early in the ethylene signalling pathway; it was suggested that the ethylene receptor has a requirement for copper and that in the partial loss of function RAN1 mutants (*ran1-1* and *ran1-2*), there is a reduction in the delivery of copper to the receptor due to the loss of function of the Cu^{2+}-ATPase. Without copper, the ligand specificity of the receptor is modified, allowing TCO to act as an agonist and activate the ethylene response pathway. The proposed role of RAN1 in copper trafficking and ethylene signalling is shown in Fig. 5.

2.5. Regulation

Since intracellular levels of heavy metals must be carefully controlled, transporters represent good candidates for regulation. As yet there are no indications of how they may be regulated in higher plants but, potentially, this could occur at the transcriptional level (control on initiation rates, mRNA stability, differential mRNA splicing) or at the post-translational level (targeting, stability). Many metal transporters in other organisms are regulated at the transcriptional level by extracellular metal concentrations via transcription factor proteins [45]. Transcriptional regulation in response to substrate has been observed for both CopA and CopB of *E. hirae*. It was discovered that two genes, *CopY* and *CopZ*, located upstream of the *copAcopB* region are metalloregulatory genes which induce expression of both ATPases in response to low and high copper concentrations [46]. Copper-specific transcriptional regulation has also been observed for copper uptake genes in yeast. MAC1 is a metal-sensitive transcription factor which regulates the transcription of CTR1 and CTR3 [47]. It is also involved in the regulation of FRE1, a metal reductase that is required for both copper and iron uptake in yeast. MAC1 recognises specific DNA sequences in upstream regions of these genes. It is negatively regulated by copper; thus in the absence of copper, MAC1 binds to these promoter elements and expression of the gene occurs. Similarly, AFT1 is an iron-dependent transcription factor that regulates the expression of the *FET3* and *FTR1* genes. The copper CPx-ATPase, CCC2, has also been shown to be regulated by AFT1 [48].

There is evidence for post-transcriptional modifi-

cation of the human Menkes and Wilson's Cu^{2+}-ATPases, with alternatively spliced isoforms that show tissue-specific expression [49,50]. One of these alternatively spliced isoforms lacks a number of regions including the β-domain, the CPx motif and the last five metal-binding domains, and is found predominantly in the cytosol [49]. It is thought that the loss of two transmembrane helices could prevent this protein from inserting into the membrane. It has been suggested that the shortened version of the Menkes ATPase may also yield a soluble protein and may have a role in copper-binding similar to that of the metal chaperone proteins ATX1 and HAH1 [50]. As yet, there is no evidence for alternative spliced isoforms of the plant CPx-ATPases.

There is evidence that the Menkes P-type ATPase encoded by *ATP7A* may continuously recycle between the Golgi and the plasma membrane in response to copper, and thus provide a novel system of regulated protein trafficking where the ligand directly stimulates the trafficking of its own transporter [51]. This ultimately leads to the efflux of an essential but potentially toxic metal [51]. Sub-cellular localisation studies following expression of ATP7A in Chinese hamster ovary cells suggests that under basal low copper conditions, this protein is localised in the *trans*-Golgi network [52]. It has been suggested that the putative copper-binding motifs in the amino-terminal region may serve as a copper-sensing domain; these sites would become progressively occupied as the cytoplasmic copper concentration rises and this may trigger the exocytotic movement of the protein to the plasma membrane [51]. For endocytosis, there may be targeting signals in the C-terminus and studies using in vitro mutagenesis may reveal which sites are important [51].

Thus the heavy metal (CPx-type) ATPases have considerable potential as key heavy metal transporters in higher plants, involved not only in normal metal ion homeostasis but also in the overall strategy of heavy metal tolerance. The cloning and functional analysis of the CPx-ATPases will give us an opportunity to study their role in metal homeostasis and the overall nutritional requirement of the plant.

3. Nramp family

3.1. Introduction

Nramp defines a novel family of related proteins which have been implicated in the transport of divalent metal ions. The *Nramp* gene family has been highly conserved during evolution and homologues have been found in a wide range of living organisms including bacteria, yeast, insects, mammals and also higher plants. The mammalian *Nramp1* was the first gene of this family to be identified. It encodes an integral membrane protein found on the endosomal/lysosomal compartment of the macrophage; upon phagocytosis, it is rapidly recruited to the phagosome membrane where it is thought to regulate the replication of engulfed bacteria by controlling divalent cation concentrations within this compartment (for a review, see [53]). Mutations in this gene result in susceptibility to infection by intracellular pathogens such as *Mycobacterium, Salmonella* and *Leishmania* [54,55].

In higher plants, investigations of the Nramp family have been largely restricted to rice, *Oryza sativa*, where three Nramp homologues have been identified. An EST from rice which showed similarity to the mammalian *Nramp1* genes was used to clone three genes, *OsNramp1*, *OsNramp2* and a partial-length *OsNramp3* [56,57]. Subsequently, Alonso et al. [58] have identified two *Arabidopsis* genes which show

→

Fig. 6. Multiple alignment of plant and mammalian Nramp protein sequences. The alignment was performed using ClustalW (1.7) sequence alignment software. An asterisk below the sequence indicates identical amino acids. Transmembrane domains (M1–M12) are underlined. Possible N-linked glycosylation sites are highlighted in grey. The conserved transport signature is highlighted in black. The accession numbers and references for the sequences aligned are: rice OsNramp1 (L41217; [56]), rice OsNramp2 (L81152; [57], rice OsNramp3 (U60767; [57]), *Arabidopsis* AtNramp1 (AC004401; unpublished), *Arabidopsis* AtNramp2 (AF141204; [58]), *Arabidopsis* AtNramp3 (AB007645; unpublished), *Arabidopsis* AtNramp4 (AL035526; unpublished), *Arabidopsis* AtNramp5 (Z30530; unpublished), mouse MmNramp1 (L13732; [59]). OsNramp3 and AtNramp5 are partial sequences. Sequences were obtained from the EMBL/GenBank databases. No protein translation was available for AtNramp4. The protein translation and gene structure were predicted using NetPlantGene [100].

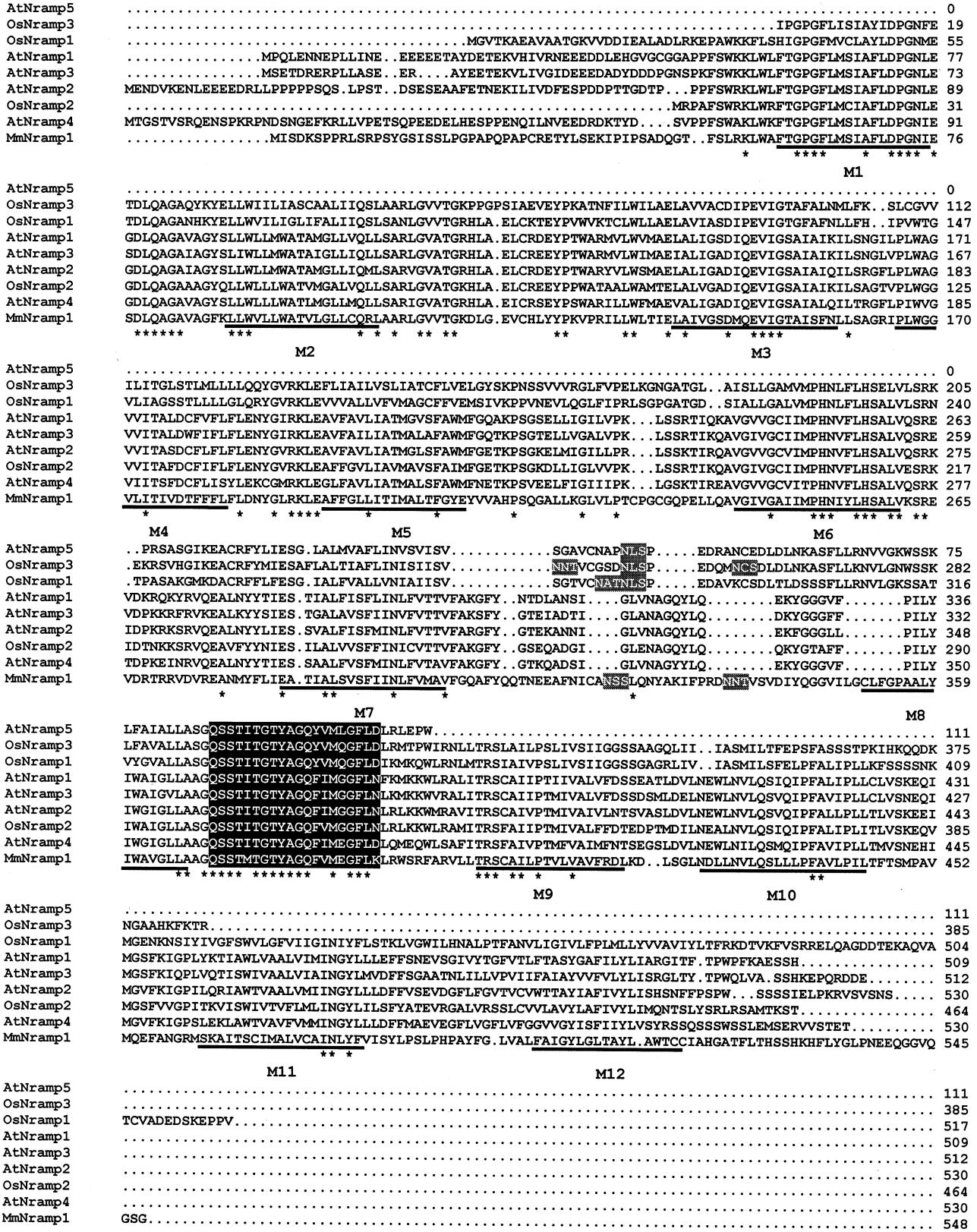

AtNramp5
OsNramp3
OsNramp1
AtNramp1
AtNramp3
AtNramp2
OsNramp2
AtNramp4
MmNramp1
M1
M2
M3
M4
M5
M6
M7
M8
M9
M10
M11
M12

Table 2
Sequence similarities between putative Nramp proteins

	AtNr-amp1	AtNr-amp2	AtNr-amp3	AtNr-amp4	AtNr-amp5[a]	OsNr-amp1	OsNr-amp2	OsNr-amp3[a]	EIN2	MmNr-amp1	DCT1	SMF1
Species	*A. thaliana*	*A. thaliana*	*A. thaliana*	*A. thaliana*	*A. thaliana*	*O. sativa*	*O. sativa*	*O. sativa*	*A. thaliana*	*Mus musculus*	*Rattus norvegicus*	*S. cerevisiae*
Substrate	nd	nd	nd	nd	nd	nd	nd	nd	nd	Fe	Fe/Mn/Zn	Mn/Cu/Cd
AtNr-amp1		74.4	76.8	66.9	44.8	40.8	70.2	40.8	26.4	49.7	50.6	31.1
AtNr-amp2	81.2		71.3	70.6	46.7	38.1	69.7	39.0	26.3	47.8	48.3	30.5
AtNr-amp3	84.1	79.4		62.4	44.8	39.0	68.6	39.8	25.1	51.3	49.7	29.8
AtNr-amp4	73.2	77.0	71.1		44.2	37.9	63.9	37.9	27.1	45.1	43.8	29.1
AtNr-amp5[a]	54.3	57.1	56.2	54.8		66.7	47.6	77.5	20.4	42.2	40.9	32.4
OsNr-amp1	50.7	49.6	51.4	49.5	76.6		38.6	61.0	25.4	35.0	38.0	35.4
OsNr-amp2	77.3	77.8	75.0	72.9	55.2	51.7		40.7	26.2	51.9	51.9	32.9
OsNr-amp3[a]	53.3	51.3	52.4	49.9	83.8	69.4	51.3		25.6	43.4	41.2	37.8
EIN2	40.6	40.3	39.2	39.6	30.1	36.4	39.7	37.2		26.0	25.1	25.3
MmNr-amp1	59.7	56.7	61.3	54.7	54.1	46.5	60.3	55.5	37.0		63.7	29.9
DCT1	61.4	59.5	60.1	54.3	53.6	49.7	60.6	54.9	36.9	70.5		31.3
SMF1	40.7	40.3	39.2	39.6	30.1	46.4	43.2	49.5	36.9	41.4	41.5	

Percentage identities (upper right triangle) and percentage similarities (lower left triangle) were calculated using the GAP program (GCG software package). Accession numbers and references for published sequences are: OsNramp1 (L41217; [56]), OsNramp2 (L81152; [57]), OsNramp3 (U60767; [57]), AtNramp2 (AF141204; [58]), EIN2 (AF141203; [58]), MmNramp1 (L13732; [59]), DCT1 (AF008439; [60]), SMF1 (U15929; [62]).
nd, not determined.
[a]Partial sequence.

similarity to *Nramps*. One of these genes, *AtNramp2*, has high similarity to the mammalian and rice *Nramps* (Table 2). A second gene called *EIN2* (ethylene-insensitive 2), which is a component in the ethylene signalling pathway, shows fairly low similarity with the *Nramp* genes (Table 2); however, there is significant similarity between EIN2 and Nramp proteins in the hydrophobic region of these proteins [58]. Our recent searches have identified three additional genomic sequences from *Arabidopsis* (Acc. No. AC004401, AB007645 and AL035526) with homology to the *Nramps*, which we have called *AtNramp1*, *AtNramp3* and *AtNramp4*. Belouchi et al. [56] also identified an EST from *Arabidopsis* (Acc. No. Z30530; called *AtNramp5*) which is a partial-length fragment of a putative *Nramp* gene which is different to the other *Arabidopsis* genes, suggesting that there are at least six homologues in this species. Comparisons of pairwise similarities between each of these genes, shown in Table 2, suggests that the plant *Nramps* can be broadly divided into two groups. *OsNramp1*, *OsNramp3* and *AtNramp5* share high sequence similarity whereas they each have lower similarity to *OsNramp2*, *AtNramp1*, *AtNramp2*, *AtNramp3* and *AtNramp4* which form another group (Table 2). This latter group also has high similarity with a partial-length EST from cotton (Acc. No. AI055379). This suggests the possibility of subgroups that may vary in their substrate specificity, although this remains to be demonstrated. Alignment of the rice and *Arabidopsis Nramp* genes with *Nramp1* from mouse is shown in Fig. 6.

3.2. Structure

As with other members of this family, the plant Nramp proteins have 12 predicted transmembrane domains (Fig. 6). EIN2 is also predicted to share this topology pattern and behaves as an integral membrane protein [58]; however, it also possesses a long intracellular C-terminal tail which is unique to the Nramp proteins. A sequence motif described as a 'consensus transport signature' [59] has been identified in the Nramp family in the fourth intracellular loop between transmembrane domain 8 and 9 ([57,60]; Fig. 6) and this is conserved amongst all the plant Nramp homologues. This transport motif bears similarities to a signature originally noted in a number of bacterial transport proteins, and particularly to a highly conserved portion of the permeation pore of the animal *shaker*-type K^+ channel [57]. This sequence appears to be the most highly conserved segment within these structures and suggests an important role in the mechanism of action of the proteins. Predicted N-linked glycosylation sites on the extracellular loop between TM 7 and 8 on OsNramp1 and OsNramp3 [57] are absent on all full-length *Arabidopsis* Nramp homologues, but are present on the *AtNramp5* sequence (Fig. 6). This may suggest differences in membrane localisation.

3.3. Physiological function

The similarity of these genes in distantly related organisms ranging from bacteria to man suggests that their proteins have a very basic physiological function that has been highly conserved throughout evolution [53]. A transport function for the plant Nramp homologues remains to be formally demonstrated; however, there is good evidence from yeast and mammalian studies for a role of the Nramp proteins in divalent cation transport. In yeast, three members of the Nramp family have been identified (SMF1, SMF2 and SMF3) [61–64], and their sequence identity with the plant proteins reaches 36% (Table 2). The physiological function of SMF1 and SMF2 is not clear although they have been proposed to function as high- and low-affinity transporters for manganese uptake, respectively [63,64]. However, they have also been shown to mediate the uptake of other metal ions such as copper [65] while SMF2 was suggested to have greater preference for cobalt [65]. Pinner et al. [55] tested whether the structural similarity between the mammalian Nramp and the yeast SMF proteins results in functional complementation by using yeast mutants bearing null alleles at the *SMF1* and *SMF2* loci. *Nramp2*, but not *Nramp1*, was found to complement hypersensitivity to both the presence of EGTA and also growth at alkaline pH, while manganese was the only divalent cation capable of suppressing both of these phenotypes. It was thus suggested that Nramp2 could transport manganese in yeast. The function of the Nramp2 homologues has recently been elucidated and evidence from a number of groups showed that it is the major transferrin-independent iron uptake system of the intestine in mammals [60,66,67]. When expressed in oocytes, DCT1 (which is the rat homologue of human Nramp2; 92% identity) is capable of transporting iron, manganese, zinc and several other divalent cations in a pH-dependent, electrogenic manner suggesting a proton-coupled carrier [60].

It has been proposed that Nramp1 may deplete the phagosome of manganese and prevent the engulfed bacteria from synthesising manganese-dependent defence enzymes such as superoxide dismutase [64]. However, since Nramp1 could not complement the SMF yeast mutants, alternative substrates have been investigated. Recently, Atkinson and Barton [68] showed that Nramp1 can modulate the accumulation of extracellular iron when expressed in COS cells. Since Nramp1 is localised to intracellular membranes [55,64], it was suggested that it may play a role in a salvage pathway of iron recycling.

The high degree of sequence similarity within the Nramp family (Table 2) suggests that the plant homologues could similarly have a metal transport function, although the metal substrates may conceivably vary between the two plant sub-groups identified by sequence similarity (Table 2). Heterologous expression of EIN2 failed to detect metal transport activity [58]. However, preliminary results from the laboratory of Julian Schroeder [69] suggest that other *Arabidopsis Nramp* homologues do encode functional metal transporters as they are able to complement a yeast Nramp knockout strain. Northern analysis indicates that OsNramp1 is expressed primarily in roots of rice, OsNramp2 mainly in leaves, and OsN-

ramp3 in both tissues [57]. Their distinct tissue expression patterns suggest there may be differences in regulation and their ability to function in certain cellular environments. Alternatively, it may suggest that they transport distinct but related ions required for particular physiological processes in different parts of the plant. This remains to be tested.

3.4. Regulation

Liu et al. [65] demonstrated that the yeast metal transport proteins SMF1 and SMF2 were under negative control by BSD2, an ER-localised protein. Their studies showed that mutation of BSD2 inactivated at least two independent metal transport systems, the uptake of cadmium and copper by SMF1 and the uptake of cobalt by SMF2. Liu et al. [65] proposed that BSD2 may act by preventing uncontrolled metal ion uptake by SMF1 and SMF2 and that BSD2 might serve as a sensor for intracellular metal pools. Subsequent work has found that BSD2 regulates SMF1 at the post-translational level [70]. Normally, SMF1 is targeted to the vacuole and then degraded; however, in *bsd2*Δ mutants, SMF1 does not enter the vacuole and the protein is stabilised. Additionally, the depletion of heavy metals from the growth medium results in accumulation of SMF1 at the plasma membrane and increased protein stability; thus both BSD2 and metal ions regulate SMF1 [70]. Whether the plant Nramp proteins are under similar control remains to be investigated.

4. CDF family

4.1. Introduction

CDF proteins are a family of heavy metal transporters implicated in the transport of zinc, cobalt and cadmium that have been identified in bacteria, archaea and eukaryotes. For a more detailed review of this family, see [22]. These proteins exhibit a high degree of sequence divergence and size variation (300–750 residues). Certain members of the CDF family are thought to function in heavy metal uptake while others catalyse efflux, and some are found in plasma membranes while others are in intracellular membranes. Some of the best characterised members of this family are the ZnT zinc efflux transporters of human and rodents and four genes have been identified to date, *ZnT-1*, *-2*, *-3* and *-4* [71–74]. Recently, a related transporter cDNA, *ZAT* (zinc transporter of *A. thaliana*; from hereon referred to as *ZAT1*), was reported [75] and our database searching of the *Arabidopsis* sequence database with the mammalian *ZnT* sequence identified two genes on two BAC clones from the genome sequencing project (Acc. Nos. AC004561 and AC005310). *ZAT1* is identical to the sequence on the AC005310 BAC clone and therefore the AC004561 sequence will be referred to hereon as *ZAT2*. An alignment of the *Arabidopsis ZAT* sequences with members of the *ZnT* family is shown in Fig. 7. *ZAT1* has highest homology to *ZnT-2* (44% identity, 69% similarity) and *ZAT2* shows highest sequence homology to *ZnT-4* (34% identity, 63% similarity) while both genes have 43% identity (67% similarity) to each other.

4.2. Structure

The mammalian *ZnT* genes encode membrane proteins with six predicted TM domains (1–6) and hydropathy analysis of the *Arabidopsis* genes indicates that both plant proteins show a similar topology. The hydropathic profile of ZAT2 compared with that of ZnT-4 is shown in Fig. 8. It has been proposed that the four amphipathic helices 1, 2, 5 and 6 constitute an inner core forming a channel while the remaining hydrophobic helices are located in the more lipid-exposed outer shell [22]. The β-carboxyls of the conserved aspartate residues in helices 2, 5 and 6 (also found in ZAT1 and ZAT2; Fig. 7) may form a cation-binding site inside the channel [22]. The plant proteins also share the CDF-specific signature sequence (Fig. 7, [22]).

In the mammalian ZnT sub-family, a multiple histidine domain is present on a long cytoplasmic loop between TM 4 and 5, which may serve as a zinc-binding region [72–74]. A comparable histidine-rich domain is also present on both *Arabidopsis* genes, and the plant proteins are noticeable longer in this region. A similar histidine-rich motif is found on many of the ZIP proteins ([43]; see Guerinot, this issue) and it is interesting to note that this histidine motif is very similar to that present on the Z99707 putative heavy metal ATPase.

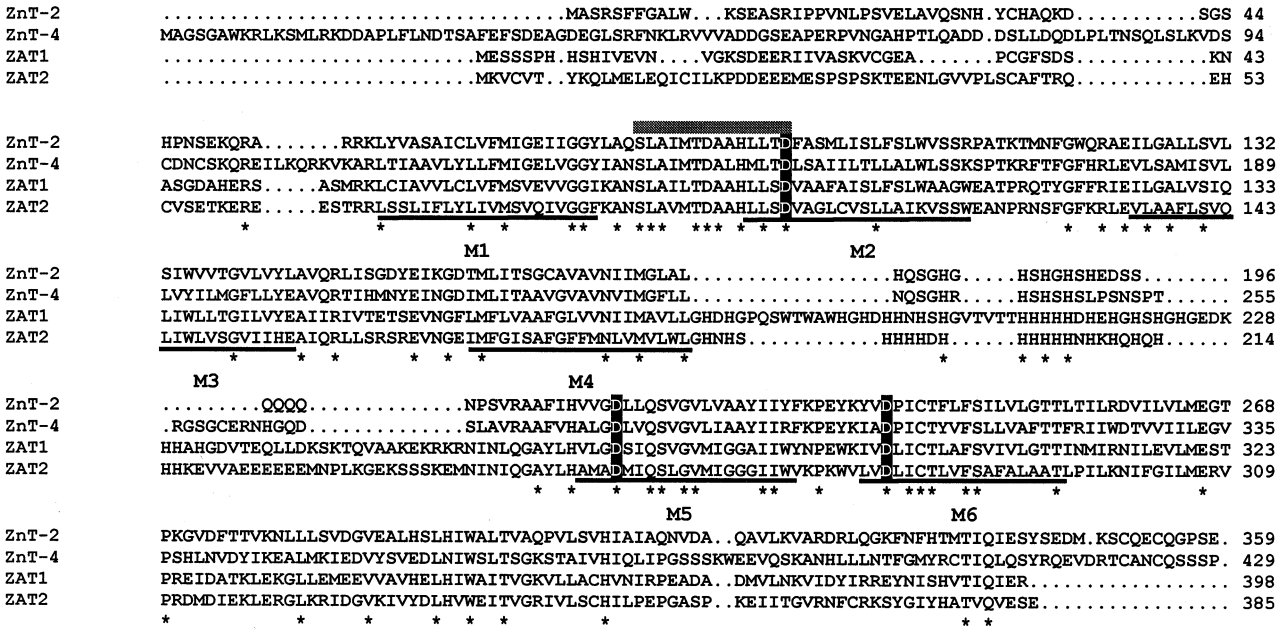


Fig. 7. Multiple alignment of the putative plant CDFs, ZAT1 and ZAT2 with members of the ZnT sub-family. The alignment was performed using ClustalW (1.7) sequence alignment software. An asterisk below the sequence indicates identical amino acids. The CDF family-specific signature sequence is denoted as a grey box above the alignment. Putative transmembrane domains (M1–6) are underlined. Conserved aspartate residues which are proposed to form a metal-binding site are highlighted in black. The accession numbers and references for the sequences aligned are: rat ZnT-2 (U50927; [72]), human ZnT-4 (AF025409) [74], *Arabidopsis* ZAT1 (AF072858; [75]), *Arabidopsis* ZAT2 (AC004561; unpublished). Sequences were obtained from the EMBL/GenBank databases.

4.3. Phylogenetic analysis

Phylogenetic analysis of the *Arabidopsis* proteins ZAT1 and ZAT2, compared with other CDF family members, shows that these proteins cluster with ZnT proteins from rat, human and *C. elegans* (Fig. 9). Other eukaryotic CDF proteins, including those from yeast which may transport other metals [76,77], form a separate cluster, while all the prokaryotic members of the family form a third sub-group (Fig. 9).

4.4. Function

The precise function of the *Arabidopsis* ZAT transporters remains to be demonstrated but the recent study by van der Zaal et al. [75] suggests that ZAT1 may have a role in zinc sequestration. Enhanced zinc resistance was observed in transgenic plants overexpressing ZAT1, and these plants showed an increase in the zinc content of the root under high zinc exposure. However, this transporter is not confined to root tissue; northern analysis in-

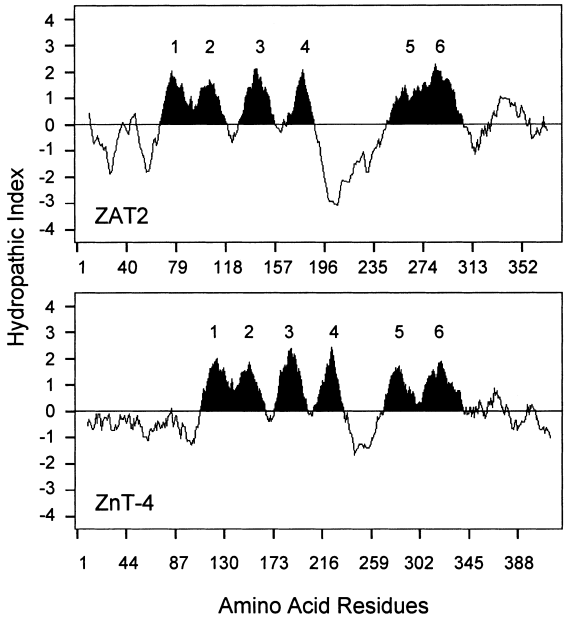


Fig. 8. Hydropathy plots of CDF protein members, ZAT2 from *Arabidopsis* and ZnT-4 from human. Plots were calculated according to Kyte and Doolittle [12] with a window size of 21 amino acids. The six predicted transmembrane domains are shaded.

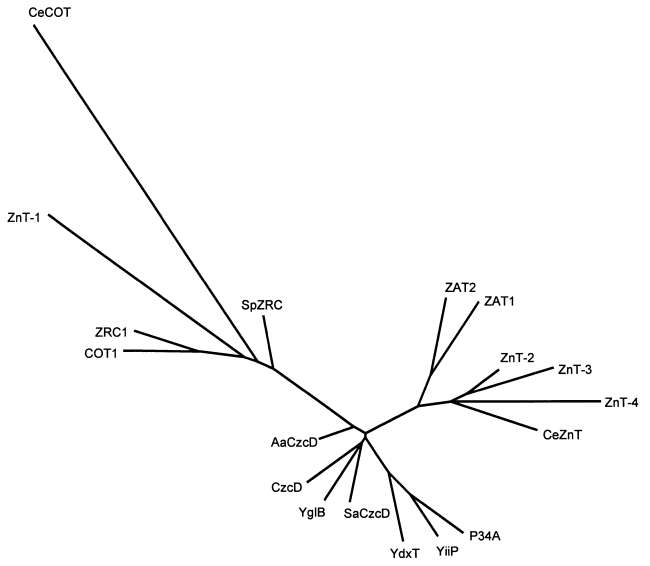


Fig. 9. Phylogenetic analysis of CDF protein members from prokaryotes and eukaryotes. The tree was constructed from alignments of full-length amino acid sequences using the Protdist and Fitch programs on the PHYLIP package [99]. The organism and accession numbers for the sequences used are: ZAT1 *Arabidopsis* AF072858, ZAT2 *Arabidopsis* AC004561, ZnT-1 rat U17133, ZnT-2 rat U50927, ZnT-3 human U76010, ZnT-4 human AF025409, CeZnT *C. elegans* Z68119, COT1 *S. cerevisiae* P32798, ZRC1 *S. cerevisiae* P20107, SpZRC *S. pombe* D89236, CeCOT *C. elegans* U23529, CzcD *Alcagenes eutrophus* P13512, AaCzcD *Aquifex aeolicus* AE000721, SaCzcD *S. aureus* AB016431, YglB *Bacillus stearothermophilus* D87026, YdxT *Bacillus subtilis* P46348, YiiP *E. coli* P32159, P34A *Rickettsia rickettsii* P21559. Sequences were obtained from the EMBL/GenBank or SwissProt databases.

dicated that ZAT1 was constitutively expressed throughout the plant and was not induced by exposure to increasing zinc concentrations. Further studies demonstrating the membrane localisation of the putative ZAT transporters would help to clarify their physiological role.

The mammalian ZnT proteins vary in their membrane location and function. ZnT-1 is present at the plasma membrane while ZnT-2 is endosomal [72]. Both of these proteins confer resistance to zinc and it appears that both are unable to transport other metals including cadmium or copper [71,72]. Some CDF proteins, however, may be involved in the transport of other metals. Two ZnT homologues have been identified in yeast; ZRC1 confers resistance to cadmium as well as zinc [76] and COT1 confers resistance to cobalt [77]. It was suggested that COT1 may function in sequestering metal ions in the mitochondria [77] but an examination of epitope-tagged protein suggests instead that COT1 and ZRC1 are localised at the vacuole [78]. It has been suggested that the CDF proteins may have a regulatory role rather than directly transporting metal ions [79], although topological studies show that four of the TM domains may form a channel-like structure [22]. The mechanisms of energy coupling are not well understood. Palmiter and Findley [71] suggest that zinc efflux mediated by the ZnT proteins should be an energy-dependent process. Since there are no nucleotide-binding domains present on the protein, the transport process may be coupled to another active transport mechanism.

5. Alternative mechanisms of metal transport in plants

As mentioned previously, a putative copper influx protein, COPT1, has been identified from *Arabidopsis* that is believed to be homologous in function to the yeast CTR1 copper transporter [42]. *COPT1* encodes a protein of 169 amino acids and has three potential TM domains. It is a smaller protein than CTR1 (28% identity) but has a methionine, histidine and serine-rich N-terminus similar to the N-terminal region of CTR1 [80] and the human homologue hCTR1 [81]. COPT1 has higher similarity to CTR2 and CTR3, which are also suggested to function as copper transporters in yeast [42,82]. Additionally, expression of COPT1 in yeast confers increased sensitivity to copper toxicity, suggesting that this protein is a copper transporter [42]. *COPT1* may be a member of a small gene family in *Arabidopsis*. Several ESTs have previously been identified which encode proteins with similarity to COPT1 [43], and we have identified another similar gene in the genome sequencing database (on BAC clone AC005623). This second gene is 71% identical to *COPT1* and is predicted to encode a protein of similar size which has the same hydropathic profile as COPT1 and a methionine, histidine and serine-rich N-terminus. COPT1 is present in flowers, stems and leaves but not in roots [42]; therefore, another homologue may be responsible for copper uptake into the roots (e.g. AC005623).

The compartmentalisation of metals into the vac-

uole is one possible mechanism that may contribute to heavy metal detoxification and tolerance. While CPx-ATPases may be candidates for transporting metals across the tonoplast, other mechanisms have been identified. It has been shown that cadmium can be transported into the vacuoles of a range of plant species [83,84]. A Cd^{2+}/H^{+} antiport mechanism has been identified in vacuolar fractions purified from oat root which has a K_m for Cd^{2+} of 5.5 μM [85]. Whether this mechanism is a lower affinity metal transporter analogous to the Ca^{2+}/H^{+} antiporter is unclear. It will need to be clarified whether the ΔpH-dependent cadmium transport that has been identified is due to a novel Cd^{2+}/H^{+} antiporter or whether cadmium is being transported by the Ca^{2+}/H^{+} antiporter. It has been suggested that *CAX1* and *CAX2* which encode putative Ca^{2+}/H^{+} antiporters from *Arabidopsis* may also transport cadmium [43]. The transport of other heavy metals by an antiport mechanism has not yet been identified. For example, it has been shown that nickel does not affect the ΔpH-dependent cadmium transport into oat root vacuoles [83].

Some metals have been shown to form complexes with other compounds within the plant cell which are then sequestered into an internal store. The transport of a Zn^{2+}-citrate complex was studied in tolerant varieties of *Silene vulgaris* where it was found that a MgATP-dependent uptake of Zn^{2+}-citrate occurred into the vacuole; at high concentrations, this transport was significantly higher in tolerant than in non-tolerant varieties [86]. Proteins have been identified in yeast which transport heavy metal chelates into the vacuole [87,88]. HMT1, a vacuolar ABC transporter, catalyses MgATP-dependent, vanadate-sensitive uptake of both Cd^{2+}-phytochelatin complexes and apo-phytochelatins [87] and, although no homologues have been found in plants, a similar activity has been observed in oat root tonoplast vesicles [89]. ScYCF1 is also a member of the ABC transporter family in yeast [90]. It confers resistance to cadmium and catalyses the MgATP-dependent transport of a cadmium-glutathione complex across the tonoplast [91]. Two homologues to this protein, AtMRP1 and AtMRP2, have been identified in *Arabidopsis* but they do not confer resistance to cadmium. However, a third protein, AtMRP3, does relieve cadmium sensitivity; therefore, it may transport cadmium complexes although this remains to be investigated ([92]; Theodoulou, this issue).

LCT1, cloned from wheat, was suggested to mediate the uptake of cadmium [93]. Expression of LCT1 in yeast showed that it was able to mediate the uptake of sodium, rubidium and calcium, as well as cadmium into the cell [93]. It was suggested that LCT1 may normally function as a mediator of calcium transport but may also transport other heavy metals. Whether LCT1 is localised at the plasma membrane or an internal membrane remains to be determined.

The best characterised heavy metal uptake transporters in higher plants to date are the ZIP family of proteins which include the IRTs (see Guerinot, this issue for detailed discussion). These have been described in a range of organisms and recently, homologues have been identified in *Arabidopsis*. The IRTs are involved in iron uptake, while the ZIPs have been shown to transport zinc [21,94]. There is also evidence that the ZIP transporters may be able to transport other metals such as manganese and cadmium [43] while IRT1 is also able to transport iron, zinc and manganese in yeast [95].

6. Summary and future perspectives

Although heavy metal ions are essential components of a variety of enzymes, transcription factors and other proteins, the mechanisms that contribute to metal ion homeostasis are only starting to be elucidated in higher plants. At the cellular level, specific transporters are presumably responsible for the uptake and secretion of metal ions, and there may be additional transporters that allow sequestration into organelles. Some of the proteins may serve a 'housekeeping' function important for cellular homeostasis while others may have more specialised roles.

We are now starting to recognise the complexity of heavy metal homeostasis with the identification of a range of proteins (including transporters) which are involved. The *Arabidopsis* genome sequencing project will be completed in the next few years and this will yield further candidate genes encoding proteins with a potential role in heavy metal transport. Work has already begun to identify the gene families responsible for metal transport and studies are now required

to distinguish their individual contribution. The transporters that we have discussed in this review (CPx-ATPases, Nramps and CDFs) belong to families already identified and studied, to variable extents, in other organisms. Thus we can utilise the information derived from these studies to augment our knowledge but at the same time, we need to recognise that there may be major differences in the range, localisation, function and regulation of the transporters in plants to accommodate their unique biological position. Thus we need to gather a range of data. For example, it will be important to determine the tissue, cell and sub-cellular localisation of the plant transporters. For this, molecular approaches such as epitope-tagging and the generation of promoter-GUS/GFP fusions will prove invaluable. The 'reverse genetics' strategy is now making an impact on the transporter field and already this approach has provided evidence that a heavy metal ATPase (AMA1) may have an important physiological role in molybdenum transport (Harper, J.F., personal communication). The availability of more mutants with T-DNA or transposon inserts in particular heavy metal transporters will allow us to monitor their specific function in vivo. The resulting 'knock-out' mutants can yield more conclusive information concerning gene function than antisense technology although the latter may also be useful.

Structure/function studies are also important for determining the mechanism of action of the different transporters. Heterologous expression systems such as yeast may prove useful for this and the possibility of purifying tagged proteins from such systems and reconstitution into artificial membranes could prove fruitful in terms of providing a system for detailed biochemical studies. It is crucial however that the information from heterologous expression systems such as yeast and *Xenopus* oocytes is verified experimentally in plants.

As mentioned above, a future challenge will be to determine how these transporters are regulated in plants. There are likely to be many tiers of control and modulation and, although there may be some similar aspects of regulation to those occurring in other organisms, unique regulatory mechanisms may also exist. The yeast two-hybrid system may be useful for identifying proteins interacting with particular domains of the transporters. In particular the interaction of metal chaperones with transporters deserves attention since this may have important implications for sequestration of metals within intracellular stores.

We need to use all this information to build a picture of how heavy metal homeostasis is achieved, recognising that there may be major differences in the operation of transporters in different species, e.g. metal accumulators and excluders. If we can define and manipulate the mechanisms by which plants take up, extrude and compartmentalise heavy metals, and also redistribute them around the plant, not only have we answered a number of fundamental questions but there are also significant biotechnological applications which can be exploited, both in terms of human nutrition and phytoremediation. Thus strategies to engineer or select plants for such applications will undoubtedly be very important.

Thus heavy metal transport is a very exciting and developing field in plant biology and we are poised at the discovery of a range of new ion transporters that will undoubtedly change our concepts of metal nutrient acquisition and homeostasis in higher plants.

Acknowledgements

This work was supported by The Royal Society and the Biotechnology and Biological Sciences Research Council.

References

[1] H. Marschner, Mineral Nutrition of Higher Plants, 2nd edn., Academic Press, London, 1995.
[2] D.T. Clarkson, U. Lüttge, Prog. Bot. 51 (1989) 93–112.
[3] F. vanAssche, H. Clijsters, Plant Cell Environ. 13 (1990) 195–206.
[4] A.A. Meharg, Plant Cell Environ. 17 (1994) 989–993.
[5] D.E. Salt, R.D. Smith, I. Raskin, Annu. Rev. Plant Physiol. Plant Mol. Biol. 49 (1998) 643–668.
[6] K.B. Axelsen, M.G. Palmgren, J. Mol. Evol. 46 (1998) 84–101.
[7] M.G. Palmgren, K.B. Axelsen, Biochim. Biophys. Acta 1365 (1998) 37–45.
[8] M. Solioz, C. Vulpe, Trends Biochem. Sci. 21 (1996) 237–241.
[9] K. Tabata, S. Kashiwagi, H. Mori, C. Ueguchi, T. Mizuno, Biochim. Biophys. Acta 1326 (1997) 1–6.

[10] T. Hirayama, J.J. Kieber, N. Hirayama, M. Kogan, P. Guzman, S. Nourizadeh, J.M. Alonso, W.P. Dailey, A. Dancis, J.R. Ecker, Cell 97 (1999) 383–393.
[11] A. Siegmund, A. Grant, C. Angeletti, L. Malone, J.W. Nichols, H.K. Rudolph, J. Biol. Chem. 273 (1998) 34399–34405.
[12] J. Kyte, R.F. Doolittle, J. Mol. Biol. 157 (1982) 105–132.
[13] J.V. Møller, B. Juul, M. leMaire, Biochim. Biophys. Acta 1286 (1996) 1–51.
[14] L. Aravind, M.Y. Galperin, E.V. Koonin, Trends Biochem. Sci. 23 (1998) 127–129.
[15] K. Maruyama, D.M. Clarke, J. Fujii, G. Inesi, T.W. Loo, D.H. MacLennan, J. Biol. Chem. 264 (1989) 13038–13042.
[16] D.M. Clarke, T.W. Loo, D.H. MacLennan, J. Biol. Chem. 265 (1990) 22223–22227.
[17] M. Solioz, A. Odermatt, J. Biol. Chem. 270 (1995) 9217–9221.
[18] A.S. Payne, J.D. Gitlin, J. Biol. Chem. 273 (1998) 3765–3770.
[19] T. Kaneko, S. Sato, H. Kotani, A. Tanaka, E. Asamizu, Y. Nakamura, N. Miyajima, M. Hirosawa, M. Sugiura, S. Sasamoto, T. Kimura, T. Hosouchi, A. Matsuno, A. Muraki, N. Nakazaki, K. Naruo, S. Okumura, S. Shimpo, C. Takeuchi, T. Wada, A. Watanabe, M. Yamada, M. Yasuda, S. Tabata, DNA Res. 3 (1996) 109–136.
[20] J.M. Berg, H.A. Godwin, Annu. Rev. Biophys. Biomol. Struct. 26 (1997) 357–371.
[21] B.H. Eng, M.L. Guerinot, D. Eide, M.H. Saier, J. Membr. Biol. 166 (1998) 1–7.
[22] I.T. Paulsen, M.H. Saier, J. Membr. Biol. 156 (1997) 99–103.
[23] S. Lutsenko, K. Petrukhin, M.J. Cooper, C.T. Gilliam, J.H. Kaplan, J. Biol. Chem. 272 (1997) 18939–18944.
[24] J. Gitschier, B. Moffat, D. Reilly, W.I. Wood, W.J. Fairbrother, Nat. Struct. Biol. 5 (1998) 47–54.
[25] D. Strausak, S. LaFontaine, J. Hill, S.D. Firth, P.J. Lockhart, J.F.B. Mercer, J. Biol. Chem. 274 (1999) 11170–11177.
[26] C. Rensing, Y. Sun, B. Mitra, B.P. Rosen, J. Biol. Chem. 273 (1998) 32614–32617.
[27] S.J. Beard, R. Hashim, J. Membrillo-Hernandez, M.W. Hughes, R.K. Poole, Mol. Microbiol. 25 (1997) 883–891.
[28] C. Thelwell, N.J. Robinson, J.S. Turner-Cavet, Proc. Natl. Acad. Sci. USA 95 (1998) 10728–10733.
[29] P. Wyler-Duda, M. Solioz, FEBS Lett. 399 (1996) 143–146.
[30] M. Solioz, J. Camakaris, FEBS Lett. 412 (1997) 165–168.
[31] I. Voskoboinik, H. Brooks, S. Smith, P. Shen, J. Camakaris, FEBS Lett. 435 (1998) 178–182.
[32] K.J. Tsai, K.P. Yoon, A.R. Lynn, J. Bacteriol. 174 (1992) 116–121.
[33] K.J. Tsai, A.L. Linet, Arch. Biochem. Biophys. 305 (1993) 267–270.
[34] C. Rensing, B. Mitra, B.P. Rosen, Proc. Natl. Acad. Sci. USA 94 (1997) 14326–14331.
[35] M.G. Palmgren, J.F. Harper, J. Exp. Bot. 50 (1999) 883–893.
[36] J.S. Valentine, E.B. Gralla, Science 278 (1997) 817–818.
[37] C. Askwith, J. Kaplan, Trends Biochem. Sci. 23 (1998) 135–138.
[38] S.-J. Lin, R.A. Pufahl, A. Dancis, T.V. O'Halloran, V.C. Culotta, J. Biol. Chem. 272 (1997) 9215–9220.
[39] D. deSilva, S. Davis-Kaplan, J. Fergestad, J. Kaplan, J. Biol. Chem. 272 (1997) 14208–14213.
[40] R. Stearmann, D.S. Yuan, Y. Yamaguchi-Iwai, R.D. Klausner, A. Dancis, Science 271 (1996) 1552–1557.
[41] E. Himelblau, H. Mira, S.-J. Lin, V.C. Culotta, L. Penarrubia, R.M. Amasino, Plant Physiol. 117 (1998) 1227–1234.
[42] K. Kampfenkel, S. Kushnir, E. Babiychuk, D. Inzé, M.V. Montagu, J. Biol. Chem. 270 (1993) 28479–28486.
[43] T.C. Fox, M.L. Guerinot, Annu. Rev. Plant Physiol. Plant Mol. Biol. 49 (1998) 669–696.
[44] Y. Sambonigi, T. Wakabayashi, T. Yoshimizu, H. Omote, T. Oka, M. Futai, J. Biochem. 121 (1997) 1169–1175.
[45] D. Radisky, J. Kaplan, J. Biol. Chem. 274 (1999) 4481–4484.
[46] A. Odermatt, M. Solioz, J. Biol. Chem. 270 (1995) 4349–4354.
[47] S. Labbé, Z. Zhu, D.J. Thiele, J. Biol. Chem. 272 (1997) 15951–15958.
[48] Y. Yamaguchi-Iwai, R. Stearman, A. Dancis, R.D. Klausner, EMBO J. 15 (1996) 3377–3384.
[49] X.L. Yang, N. Miura, Y. Kawarada, K. Terada, K. Petrukhin, C.T. Gilliam, T. Sugiyama, Biochem. J. 326 (1997) 897–902.
[50] M.C.M. Reddy, E.D. Harris, Biochem. J. 334 (1998) 71–77.
[51] M.J. Petris, J.F.B. Mercer, J.G. Culvenor, P. Lockhart, P.A. Gleeson, J. Camakaris, EMBO J. 15 (1996) 6084–6095.
[52] S. LaFontaine, S.D. Firth, J. Camakaris, A. Englezou, M.B. Theophilos, M.J. Petris, M. Howie, P.J. Lockhart, M. Greenough, H. Brooks, R.R. Reddel, J.F.B. Mercer, J. Biol. Chem. 273 (1998) 31375–31380.
[53] G. Govoni, P. Gros, Inflamm. Res. 47 (1998) 277–284.
[54] M. Cellier, A. Belouchi, P. Gros, Trends Genet. 12 (1996) 201–204.
[55] E. Pinner, S. Gruenheid, M. Raymond, P. Gros, J. Biol. Chem. 272 (1997) 28933–28938.
[56] A. Belouchi, M. Cellier, T. Kwan, H.S. Saini, G. Leroux, P. Gros, Plant Mol. Biol. 29 (1995) 1181–1196.
[57] A. Belouchi, T. Kwan, P. Gros, Plant Mol. Biol. 33 (1997) 1085–1092.
[58] J.M. Alonso, T. Hirayama, G. Roman, S. Nourizadeh, J.R. Ecker, Science 284 (1999) 2148–2152.
[59] S.M. Vidal, D. Malo, K. Vogan, E. Skamene, P. Gros, Cell 73 (1993) 469–485.
[60] H. Gunshin, B. MacKenzie, U.V. Berger, Y. Gunshin, M.F. Romero, W.F. Boron, S. Nussberger, J.L. Gollan, M.A. Hediger, Nature 388 (1997) 482–488.
[61] G. Hawlitschek, H. Schneider, B. Schmidt, M. Tropschug, F.U. Hartl, W. Neupert, Cell 53 (1988) 795–806.
[62] A.H. West, D.J. Clark, J. Martin, W. Neupert, F.U. Hartl, A.L. Horwich, J. Biol. Chem. 267 (1992) 24625–24633.
[63] F. Supek, L. Supekova, H. Nelson, N. Nelson, Proc. Natl. Acad. Sci. USA 93 (1996) 5105–5110.
[64] F. Supek, L. Supekova, H. Nelson, N. Nelson, J. Exp. Biol. 200 (1997) 321–330.

[65] X.F. Liu, F. Supek, N. Nelson, V.C. Culotta, J. Biol. Chem. 272 (1997) 11763–11769.
[66] M.D. Fleming, C.C. Trenor, M.A. Su, D. Foernzler, D.R. Beier, W.F. Dietrich, N.C. Andrews, Nat. Genet. 16 (1997) 383–386.
[67] M.D. Fleming, M.A. Romano, M.A. Su, L.M. Garrick, M.D. Garrick, N.C. Andrews, Proc. Natl. Acad. Sci. USA 95 (1998) 1148–1153.
[68] P.G.P. Atkinson, C.H. Barton, FEBS Lett. 425 (1998) 239–242.
[69] S. Thomine, W. Rongchen, N.M. Crawford and J.I. Schroeder, Plant Biology '99 Meeting Abstract No. 772, http://www.rycomusa.com/aspp1999/public/P47.html.
[70] X.F. Liu, V.C. Culotta, J. Biol. Chem. 274 (1999) 4863–4868.
[71] R.D. Palmiter, S.D. Findley, EMBO J. 14 (1995) 639–649.
[72] R.D. Palmiter, T.B. Cole, S.D. Findley, EMBO J. 15 (1996) 1784–1791.
[73] R.D. Palmiter, T.B. Cole, C.J. Quaife, S.D. Findley, Proc. Natl. Acad. Sci. USA 93 (1996) 14934–14939.
[74] L.P. Huang, J. Gitschier, Nat. Genet. 17 (1997) 292–297.
[75] B.J. vanderZaal, L.W. Neuteboom, J.E. Pinas, A.N. Chardonnens, H. Schat, J.A.C. Verkleij, P.H. Hooykaas, Plant Physiol. 119 (1999) 1047–1056.
[76] A. Kamizono, M. Nishizawa, Y. Teranishi, K. Murata, A. Kimura, Mol. Gen. Genet. 219 (1989) 161–167.
[77] D.S. Conklin, J.A. McMaster, M.R. Culbertson, C. Kung, Mol. Cell. Biol. 12 (1992) 3678–3688.
[78] L. Li, J. Kaplan, J. Biol. Chem. 273 (1998) 22181–22187.
[79] D.H. Nies, J. Bacteriol. 174 (1992) 8102–8110.
[80] A. Dancis, D.S. Yuan, D. Haile, C. Askwith, D. Eide, C. Moehle, J. Kaplan, R.D. Klausner, Cell 76 (1994) 393–402.
[81] B. Zhou, J. Gitschier, Proc. Natl. Acad. Sci. USA 94 (1997) 7481–7486.
[82] S.A.B. Knight, S. Labbé, L.F. Kwon, D.J. Kosman, D.J. Thiele, Genes Dev. 10 (1996) 1917–1929.
[83] G.E. Gries, G.J. Wagner, Planta 204 (1998) 390–396.
[84] J.J. Hart, R.M. Welch, W.A. Norvell, L.A. Sullivan, L.V. Kochian, Plant Physiol. 116 (1998) 1413–1420.
[85] D.E. Salt, G.J. Wagner, J. Biol. Chem. 268 (1993) 12297–12302.
[86] J.A.C. Verkleij, P.L.M. Koevoets, M.M.A. Blake-Kalff, A.N. Chardonnens, J. Plant Physiol. 153 (1998) 188–191.
[87] D.F. Ortiz, T. Ruscitti, K.F. McCue, D.W. Ow, J. Biol. Chem. 270 (1995) 4721–4728.
[88] Z.S. Li, M. Szczypka, Y.P. Lu, D.J. Thiele, P.A. Rea, J. Biol. Chem. 271 (1996) 6509–6517.
[89] D.E. Salt, W.E. Rauser, Plant Physiol. 107 (1995) 1293–1301.
[90] P.A. Rea, Z.S. Li, Y.P. Lu, Y.M. Drozdowicz, E. Martinoia, Annu. Rev. Plant Physiol. Plant Mol. Biol. 49 (1998) 727–760.
[91] Z.S. Li, Y.P. Lu, R.G. Zhen, M. Szczypka, D.J. Thiele, P.A. Rea, Proc. Natl. Acad. Sci. USA 94 (1997) 42–47.
[92] R. Tommasini, E. Vogt, M. Fromenteau, S. Hörtensteiner, P. Matile, N. Amrhein, E. Martinoia, Plant J. 13 (1998) 773–780.
[93] S. Clemens, D.M. Antosiewicz, J.M. Ward, D.P. Schachtman, J.I. Schroeder, Proc. Natl. Acad. Sci. USA 95 (1998) 12043–12048.
[94] D. Eide, M. Broderius, J. Fett, M.L. Guerinot, Proc. Natl. Acad. Sci. USA 93 (1996) 5624–5628.
[95] Y.O. Korshunova, D. Eide, W.G. Clark, M.L. Guerinot, H.B. Pakrasi, Plant Mol. Biol. 40 (1999) 37–44.
[96] C. Vulpe, B. Levinson, S. Whitney, S. Packman, J. Gitschier, Nat. Genet. 3 (1993) 7–13.
[97] D. Fu, T.J. Beeler, T.M. Dunn, Yeast 11 (1995) 283–292.
[98] F. Liang, K.W. Cunningham, J.F. Harper, H. Sze, Proc. Natl. Acad. Sci. USA 94 (1997) 8579–8584.
[99] J. Felsenstein, Cladistics 5 (1989) 164–166.
[100] S.M. Hebsgaard, P.G. Korning, N. Tolstrup, J. Engelbrecht, P. Rouze, S. Brunak, Nucleic Acids Res. 24 (1996) 3439–3452.
[101] J. Chelly, Z. Tumer, T. Tonnesen, A. Petterson, Y. Ishikawabrush, N. Tommerup, N. Horn, A.P. Monaco, Nat. Genet. 3 (1993) 14–19.